STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

THE WORK OF THE STAIN COMMISSION

Organization of the Commission.—Nearly all readers of Stain Technology are familiar with the organization of the Biological Stain Commission. It has been explained more than once in statements published in these pages. Nevertheless, it seems well to repeat a little to recall its history to the reader's mind, and to explain again the present form of the organization.

The work from which the Stain Commission started was begun in 1920 as an activity of the Committee on Bacteriological Technic of the Society of American Bacteriologists. This Committee had been asked to investigate the stains available on the American market after World War I and to determine which sources were most reliable. This proved to be quite an undertaking and it soon became evident that certain other branches of science besides bacteriology would have to be involved. As a result, the National Research Council was approached on the matter and they became sufficiently interested to appoint a committee representing botany and zoology as well as bacteriology. Two members of this original committee, Dr. S. I. Kornhauser and the writer, became members of the Commission when it was first organized, and are still active in the work.

The Commission on the Standardization of Biological Stains, as it was then named, was organized in 1922, the committee appointed by the National Research Council becoming the Executive Committee of the organization. The Commission had on its membership representatives named by certain national scientific societies and was in general regarded as a coordinating committee of these organizations.

In general this remained the form of organization until 1944. The Commission had no official recognition nor any corporate existence. It was purely an informal organization of scientists interested in staining, and it had no authority other than that derived from the prestige of the scientists composing it and of the societies which they represented. In spite of this informal organization, its recommendations came gradually to have more and more weight and

the Commission-certified stains acquired more and more prestige. Finally in 1942, it secured a certain amount of official recognition when the 7th edition of the National Formulary called for the use of Commission certified stains in making up staining solutions described in that publication.

As the National Formulary specifications, in which Commission certification was called for, were available in preliminary form even before the publication date of the 7th edition, they were at the disposal of the Army and Navy purchasing agencies at the beginning of World War II. These specifications were employed by the purchasing agencies in question, and the result has been that the Army and Navy have been calling for Commission certification of biological stains during the present war.

Such recognition as this made it seem desirable to place the Stain Commission on a more permanent basis rather than have it continue as a mere informal organization, which might well go to pieces if one or two of its most active members should die or have to drop the work. Accordingly, in 1943, application was made to the Board of Regents of New York State for its incorporation under the University of the State of New York. This charter was granted on February 21, 1944; the Commission was incorporated under the name Biological Stain Commission, Inc. The by-laws of the new organization thus formed have already been published in these pages.

At present there are 58 members of the Biological Stain Commission; most of them in the United States and Canada but a few from other countries in this hemisphere who have been especially invited to represent their countries. Membership is by invitation only; nevertheless, those interested in the work, who think membership would be desirable, are asked to get in contact with the writer and let him know of their interest. Such correspondence very often leads to membership later.

The certification plan.—When the first investigations were made of the sources of stains available in this country, it was hoped that it might be possible to approve the entire product of some one or two concerns. This idea was derived very naturally from the knowledge that before the first World War the stains of one particular German firm had been regarded as standard. The earlier investigations of the Commission, however, soon showed that it would not be possible to standardize on any one manufacturer's products; that none of them, domestic or foreign, were entirely reliable. It was then hoped that specifications of a chemical or optical nature (or both) could be drawn up and products approved which con-

formed to these specifications. This was soon found to be almost equally impossible. There proved to be very little correlation between performance and the chemical or optical nature of a dye so far as it could be determined at the time. This is still true today in the case of many stains, although in some instances it is possible to determine very accurately the quality of a stain by means of spectrophotometry. Although specifications for stains have been drawn up in the National Formulary with the coöperation of the Stain Commission, these specifications always include some test as to the performance of the samples in one or more actual staining procedures. It is only by means of a test for performance that a satisfactory lot of stain can be judged.

When requirements as to performance have to be included in specifications, the tests must be made by skilled technicians. It is then obvious that the stain manufacturers cannot be supplied with specifications which they can be expected to follow and thus keep their entire product on a plane of high dependability. Hence the only practical plan the Commission could devise was for a company to submit samples of each batch of any stain and for the Commission to grant certification after testing the samples.

In other words, the certification plan that has been adopted has been put into effect on a batch basis. Any company preparing a batch of stain and desiring the Commission certification is expected to submit a sample of the batch to the Commission for testing sufficiently in advance of the market needs to allow for the necessary tests to be made. These tests always include: an assay to determine total dye content; a spectrophotometric analysis; tests as to performance in the biological procedures for which the stain is ordinarily employed. Under the last category, sometimes tests are made in one procedure only, sometimes two or three, and occasionally in more staining procedures. In passing upon a sample to decide whether to certify it, greater stress is always laid on performance than on the chemical and photometric tests.

The effect of certification.—The certification plan was adopted in 1923, one or two stains being put on the certification basis at a time. As a result, it was not until 1925 that the majority of biological stains were put on this basis. When the plan was adopted, some manufacturers fell in with the scheme quite enthusiastically; others were more skeptical. Furthermore, it took some time for users of stains to become familiar with the work of the Commission and to call for products bearing the Commission certification. As a result of these facts, it was not until about 1930 that the plan was

well under way and certified stains could be regarded as having become a staple on the American market.

This is reflected in part by the number of samples submitted to the Commission for testing as shown in Table 1. In this Table, the first two years are placed by themselves, because at that time very few dyes were on the certification basis and the work of certifying them was still on somewhat of an experimental footing. After these first few years, the remaining data in Table 1 are grouped in five-year periods. It will be seen that the number of samples sub-

TABLE 1. STAIN SAMPLES REJECTED IN RELATION TO TOTAL NUMBER TESTED

Year	Number of Samples Tested	Number of Samples Rejected	Percent of Samples Rejected	
			Per year	Per 5 year periods
1923	20	9	45%	
1924	46	14	30	
1925	39	5	13	
1926	45	3	7	
1927	57	7	12	
1928	43	2	5	
1929	42	4	10	9.3
1930	32	0	0	
1931	52	3	6	
1932	37	8	22	
1933	27	1	4	
1934	43	2	4.5	7.3
1935	34	0	0	
1936	40	4	10	
1937	31	2	6.5	
1938	51	3	6	
1939	50	7	14	7.8%
1940	71	3	4	
1941	111	5	4.5	
1942	157	9	5.7	
1943	82	5	6	
1944	79	10	12.5	6.4

mitted for testing tended to decrease until 1933 and then began to increase again, reaching a maximum in 1942, soon after the United States entered the war. These figures, of course, do not indicate the actual quantity of certified stains on the market because there was a tendency for the companies to produce larger batches as the demands for these stains became greater.

They are nevertheless interesting, showing that the number of batches of stains submitted for testing during one of the war years was three times the number submitted in any year before the beginning of the war.

Another very interesting fact brought out in the table relates to the number of samples rejected; this is given in the second column in actual numbers and in the two last columns in percentages. The percentage of rejections was high during the first two years (while the procedure was on an experimental basis), but then fell rapidly and averaged only 9.3% for the five-year period from 1925 to 1929. It fell to 7.3% during the next five-year period; increased slightly during the following period but during the last five-year period decreased[1] to 6.4%. This decrease in rejection is particularly gratifying, showing the effect that Commission certification has had on the quality of stains. During the 20 years involved, the standards of the Commission have been becoming more rather than less severe; nevertheless, the manufacturers have turned in a greater percentage

TABLE 2. CERTIFICATION LABELS ORDERED BY STAIN COMPANIES YEAR BY YEAR

Year	No. of labels	Year	No. of labels
1923	2,450	1935	21,935
1924	4,010	1936	26,770
		1937	26,338
1925	13,909	1938	29,817
1926	22,205	1939	33,640
1927	14,822		
1928	14,840	1940	67,085
1929	13,425	1941	140,868
		1942	546,106
1930	14,910	1943	510,415
1931	19,850	1944	103,139
1932	12,810		
1933	15,142	1st half of 1945	150,230
1934	18,820		

of acceptable samples during the last part of the time than during the first. This seems to be particularly significant, considering the large number of batches submitted in 1940 and the stress of war conditions under which those samples must have been manufactured.

The demand for certified stains.—The figures given above as to the number of batches of stain certified during the twenty odd years the work has continued, suggest that there must have been an increased demand for certified stains during that period. Since, however, the size of the batches has varied greatly, it is possible to get much stronger evidence from other figures. Those, for in-

[1]It will be noticed that this low average for the last five years has occurred in spite of an increase to 12.5% in 1944. This increase is regarded as very temporary; it was due to difficulties of manufacture, that have now been largely overcome, but were the result of the unavailability of certain "intermediates" during the last year of the war in Europe.

stance, which give the numbers of certification labels ordered by the companies each year are much more to the point. They are given in Table 2. Even these figures do not indicate exactly the relative amounts of dye ordered each year because certification labels are sometimes placed on small bottles, sometimes on large ones. The figures do give a rough idea of sales of certified stains, however, and therefore of the demand for these products. They seem to show that the demand remained about constant during the period from 1925 to 1935. After that it increased rapidly, especially during the war. During 1942 and 1943, each year saw about 25 times as many certification labels ordered by manufacturers as the average yearly number before 1925.

TABLE 3. CERTIFICATION LABELS ORDERED BY STAIN COMPANIES DURING THE LAST SIX YEARS

	1939	1940	1941	1942	1943	1944
Acid Fuchsin	725	2,575	3,118	12,494	8,670	4,605
Basic Fuchsin	3,400	7,440	14,660	48,897	43,300	6,722
Bismarck Brown Y	200	1,300	3,730	20,963	16,000	150
Brilliant Green	450	1,450	2,300	19,048	33,200	355
Crystal Violet	6,635	11,625	26,660	121,773	148,225	2,225
Eosin Y	1,250	2,900	5,600	19,111	17,850	2,550
Giemsa Stain	2,110	2,350	7,644	39,750	26,475	24,725
Malachite Green	700	950	1,272	21,460	6,175	3,075
Methyl Violet 2B	650	2,300	400	21,250	9,250	8,350
Methylene Blue	3,740	6,100	27,160	69,977	53,250	3,725
Safranin O	1,200	4,200	8,600	30,031	18,665	15,135
Thionin	200	1,050	1,100	4,300	6,400	2,000
Totals for above 12 stains	21,260	44,240	102,244	429,054	387,460	73,617
40 other certified stains	12,380	22,845	38,624	107,052	119,989	29,522
Grand totals	33,640	67,085	140,868	536,106	507,449	103,139

There can hardly be any question but that this demand was stimulated by the war. The moderate increase in 1940 and 1941 was apparently due to national defense measures during the period before Pearl Harbor, while the even greater increase in 1942 and 1943 was clearly to supply the needs of the Armed Forces. Correspondence with the stain companies shows that this indeed was the case; by far the great majority of orders during that period coming from the Army and Navy.

The influence of the war on the demand for certified stains is illustrated to an even greater extent by Table 3. In this Table are listed the number of certification labels ordered by stain companies during the last 6 years with 12 of the most commonly used stains listed separately. It will be seen that these 12 stains are to a very

large extent those most commonly used in bacteriology and pathology and, accordingly, are the ones most likely to be needed by the medical branches of the Service. In 1939, these 12 stains accounted for less than ⅔ of the total number of certification labels. In 1942 and 1943, however, when the total number of labels ordered had increased from 33,000 to over 500,000 a year, these 12 stains accounted for about 4/5 of the total number of labels. The decrease in the figures for 1944 are striking, and apparently indicate that after two years of intensive buying of stains, the Army and Navy must have been pretty well stocked up, and the sales of these products, therefore, fell off sharply. It is a little interesting (although the significance of the fact is obscure) that in the case of two stains, Giemsa stain and safranin, orders continued in 1944 at practically as high a rate as during 1942 and 1943. It is also noticeable that the numbers of labels furnished for the 40 stains that are not listed separately had fallen back almost to the pre-war level, while about 2 or 3 times as many labels were furnished for the 12 stains listed separately—not 4 times, as in 1942. These figures indicate quite plainly which stains are in special demand during war time; and this information together with that in Table 2 implies that these products play a significant part in war economy.

Post-war Plans.—After this war, it is hardly to be expected that the activities of the Stain Commission will be at all similar to what they were after the first World War. Then, the object was to secure uniformity and high quality in the dyes used for microscopic work. Now, that object has been largely accomplished; but others remain. First in importance is the stimulation of research in regard to stains and staining, looking toward the development of new stains and new methods. To accomplish this it has even been suggested that the Commission establish a research institute of its own to which problems of that nature could be referred. Secondly, there still remains the problem of improving staining technic and standardizing the methods. A preliminary step in the latter direction has been undertaken in publishing the laboratory manual known as "Staining Procedures", in which the staining methods used by Commission members are detailed in a standardized form. This is still to be regarded as only a preliminary step; for Staining Procedures is a loose-leaf publication consisting of several leaflets, each dealing with a particular phase of microtechnic; and it is planned to revise it section by section so as to keep the methods up to date. This alone looks like an undertaking that may well occupy a considerable part of someone's spare time.

Also, it must not be forgotten that there are still dyes that have not been standardized. A few comparatively new dyes (e. g. Sudan black B), put on the market just before the war, are still sufficiently unknown chemically so that their standardization is difficult. Then there are the natural dyes that have not yet been synthesized. Some of these, like carmine and hematoxylin, seem to be sufficiently standardized for practical purposes; but others are not. The two closely related dyes or indicators, litmus and orcein, still remain somewhat mysterious; a synthetic orcein is available, but it is not exactly like the natural product—while it has not yet proved possible to prepare litmus successfully nor to obtain in this country a natural litmus like the product available from Europe before the war.

In other words problems still remain, and it is hoped that the Stain Commission will fit into the post-war picture in such a way as to help solve them.—H. J. Conn.

FROZEN SECTIONS FROM BOUIN-FIXED MATERIAL IN HISTOPATHOLOGY

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It has become almost a dogma that, for obtaining satisfactory frozen sections, fixation of the tissues in formalin is necessary; formalin is exclusively recommended for this purpose in the textbooks of microscopical and pathological technic.[1] It has, however, several important disadvantages, which become the more acutely felt when fixation in more than one fixative is impossible, as happens so often in surgical pathology. When, after the preparation of the frozen sections, embedding in paraffin is necessary, formalin-fixed tissues show a marked shrinkage, especially when rapid embedding methods (e. g. dioxane) are used. This shrinkage can only be prevented by the time-consuming hardening of the blocks in $K_2Cr_2O_7$. The trichrome stains, the phosphotungstic-hematoxylin stain and the azan method, which are slowly superseding the hematoxylin-eosin and van Gieson stains in histopathology, give only mediocre or inferior results after formalin fixation, unless the sections are refixed.

It is commonly stated that formalin gives the tissues the consistency most suited for the preparation of frozen sections. When, however, boiling of the tissues in the fixative has to be avoided and when the specimens are in it for only a few hours (a situation which is often the case in surgical clinics, where the specimens removed during the morning are brought to the laboratory,) the consistency is generally far from ideal. Finally, when frozen sections of formalin-fixed material are mounted in glycerin-gelatine to save time or in order to avoid the extraction of fatty substances, they often fail to show the sharp definition of histological detail, which is so important in diagnostic work.

There exist several fixatives other than formalin which are suited

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[1]Cowdry, E. V. 1943. Microscopic Technique. Williams and Wilkins, Baltimore, Md. *See p. 80.*

Mallory, F. B. 1938. Pathological Technique. W. B. Saunders Company, Philadelphia and London. *See p. 35.*

Bensley, R. R., and Bensley, S. H. 1941. Handbook of Histological and Cytological Technique. University of Chicago Press, Chicago, Ill. *See p. 137.*

Krajian, Aram A. 1936. Histological Technic, *1st Ed.* Krajian, Los Angeles, Cal. *See p. 42.*

McClung, C. E. 1937. Handbook of Microscopical Technique. Paul B. Hoeber, Inc., New York, N. Y. *See p. 32.*

for the frozen section technic and for paraffin-embedding, e. g. the sublimate-formaldehyde mixture and Heidenhain's Susa mixture. They have, however, the disadvantage that in some way the tissues must be washed to remove the sublimate and that the sections must be treated with Lugol's solution and $Na_2S_2O_3$ in order to remove the precipitates of mercury. After fixation in Zenker-formol the frozen section technic gives brilliant results[2] but prolonged washing of the tissues in running water is necessary, the precipitates of mercury have to be removed and, which in pathologic histology is very important, ensuing paraffin embedding causes much shrinkage.

Bouin's fluid (prepared according to Masson's formula[3]) has none of the disadvantages mentioned and has been used in our laboratory for the frozen section technic during many years. At room temperature thin blocks of tissue are fixed in a few hours and, according to our experience, the consistency of the tissues is firmer and better suited for the frozen section technic than after fixation in formalin, so that even friable tissue cuts fairly well. Also, tissues fixed in Bouin's fluid can be handled with metallic instruments without any danger. Paraffin embedding of Bouin-fixed tissues causes relatively little shrinkage. Many microscopical details, especially those important for a histopathological diagnosis, such as mitotic figures, fibroglia, myofibrils and the structures of epithelium, are much better preserved by Bouin's fluid than by formalin, whereas after paraffin embedding nearly all staining methods give excellent results.

After the fixation is complete, the blocks of tissue are superficially washed for a few seconds in tapwater and thereafter frozen and sectioned. The sections as cut are transferred to water, washed a few seconds in 50% alcohol containing a few drops of a saturated aqueous solution of Li_2CO_3 (20 drops in 100 ml. alcohol), again transferred to water and floated on the slide. They are stained with acidulated Harris' hematoxylin, again washed in water and mounted in one of the glycerin-gelatine mixtures. (We prefer glycerin-gelatine prepared according to the formula of Grosz-Romeis[4])

In this way sections can be prepared in a few minutes. They show excellent detail, the cells contrasting with the unstained connective tissue. Myelin sheaths of peripheral nerves are stained purplish-blue. The staining of fat with Sudan III gives very good results.

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[2]Hartz, P. H. 1931. Serosadeckzellern und Hystiocyten. Zts. Zellforsch. mikr. Anat., **13**, 161–8.

[3]Masson, P. 1923. Tumeurs-Diagnostics Histologiques. A. Maloine Fils, Paris. *See p. 632–3.*

[4]Romeis, B. 1932. Taschenbuch der Mikroskopischen Technik, *13th Ed.* R. Oldenbourg, München and Berlin. *See p. 234.*

SUBSTITUTES FOR ETHYL ALCOHOL[1]

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Abstract.—**Three substitute solvents were investigated as substitutes for ethanol in nine commonly used staining fluids. Anhydrous methanol ("Bioloid" grade, Will Corporation) was found an entirely satisfactory substitute in these fluids, and isopropanol only slightly inferior.**

Some time ago one of the writers[2] called attention to the fact that a mixture of isopropanol and methanol would serve as a satisfactory decolorizing fluid in the Gram stain, in place of ethanol. The principle involved in this substitution is that in this procedure the three alcohols in question vary in speed of their decolorizing action in inverse proportion to their molecular weight; as a result equal parts of the highest and lowest of the series decolorizes at essentially the same rate as the middle member, i. e. ethanol. In this particular technic the speed of the decolorization is important, because with a decolorizer that is too slow there is a tendency for Gram-negative organisms to appear positive, while with too rapid a decolorizer, the opposite tendency may be observed.

There does not seem to be any record in the literature as to whether the same relation between the three alcohols holds in the differentiation of histological material, but it is altogether probable that it does. It is known that either of the other alcohols may be employed in place of ethanol, although they have not been widely used and no definite statements are at hand as to how to allow for differences in rate of action.

The greatest demand for a substitute for ethyl alcohol came during the period of American prohibition, when it was sometimes a difficult ingredient for small laboratories to obtain. Since that period there has been less reason for such a substitute, although it still happens at times that the excise taxes involved, except in the cases of laboratories that can buy tax-free alcohol, are sufficient to constitute an undesirable expense.

Recently, for example, one of the stain manufacturers called attention to large sums of money spent by them for the alcohol called

[1]Journal Paper No. 648, New York State Agricultural Experiment Station, Geneva, N. Y., Oct. 15, 1945.

[2]Conn, H. J. 1928. A new substitute for ethyl alcohol in the Gram stain. Stain Techn., **3**, 71–2.

for in preparing staining solutions, and asked whether it would be possible to substitute a tax-free alcohol in any of the common formulae. The tax-free alcohols they suggested were isopropanol, anhydrous methanol and anhydrous 2B alcohol (a denatured ethanol). As no answer was available from information in the literature a comparison was made.

Four sets of the following nine solutions were made up, some by the above mentioned stain manufacturer and some in the writers' laboratories:

1. Delafield's hematoxylin
2. Eosin Y, saturated alcoholic
3. Methylene blue, saturated alcoholic
4. Loeffler's methylene blue
5. Gentian violet, saturated alcoholic
6. Carbol gentian violet
7. Stirling's gentian violet
8. Basic fuchsin, saturated alcoholic
9. Ziehl Neelsen's carbol fuchsin

The four sets differed from each other in the alcohol used as a solvent, which was respectively, 95% ethanol, anhydrous 2B alcohol, anhydrous methanol or isobutyl alcohol. There were thus 36 individual solutions to test.

Tests were made in certain staining procedures in which the solutions are frequently used; before doing so, of course, the four saturated alcohol solutions (i. e., Nos. 2, 3, 5, and 8, above) were made up into the appropriate staining formula. In each of the four sets the nine solutions were tested in the following procedures:

Solutions 1 and 2 in the hematoxylin-eosin tissue stain.
Solutions 3 and 4 for staining diphtheria organisms.
Solutions 5, 6 and 7 in the Gram stain.
Solution 8 in the Endo culture medium.
Solution 9 for staining the tubercle organism.

The results can be summed up briefly by saying that both the anhydrous 2B alcohol and the anhydrous methanol proved as satisfactory in every instance as the 95% ethyl alcohol. The isopropyl alcohol was often equally good, although solutions 1 to 4 made up with it did not give quite so good results as with the other two solvents.

One very unexpected result was obtained in the case of carbol fuchsin. When prepared by the formula given in Staining Procedures[3] and in the Manual of Methods for Pure Culture Study of

[3]Conn, H. J. and Darrow, Mary A. 1943–5. Staining Procedures used by the Biological Stain Commission. Biotech Publications, Geneva, N. Y.

Bacteria,[4] results were as above indicated, that is equally good with all three solvents. When, however, it was prepared by a slightly different formula, calling for a higher percentage of fuchsin and a lower percentage of phenol, results were, surprisingly, distinctly better with either of the substitutes than with 95% ethanol. No explanation was obtained as to the reason for this unexpected difference.

Another unexplained observation is that better staining fluids were obtained when using "Bioloid" grade anhydrous methanol from the Will Corporation than with the presumably purer "Analyzed" grade of methanol C. P. from Baker Chemical Co. Whatever the explanation may be, it is obvious that the latter, more expensive, grade of this solvent is entirely unnecessary for the present purpose.

It is suggested by these results that any user of stains who finds it difficult to obtain pure grain alcohol need have no fear as to his results if he substitutes anhydrous methanol for it. Anhydrous 2B alcohol gives as good results, but is not so easy to obtain commercially. The results, to be sure, apply only to the staining solutions and technics mentioned in this note, but considering their uniformity it seems quite probable that the same situation would be true with many if not all other staining procedures calling for alcohol as a solvent for the dye.

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[4]Committee on Bacteriological Technic. 1923–35. Manual of Methods for Pure Culture Study of Bacteria. Biotech Publications, Geneva, N. Y.

A TISSUE BASKET FOR PARAFFIN INFILTRATION[1]

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The processes of washing, dehydration and paraffin infiltration when large numbers of tissues must be individually identified are always laborious. Washing and dehydration can be carried out in individual glass vials with relative ease and fairly rapidly if gravity flow reagent bottles are used to fill the vials. The use of similar vials for paraffin infiltration is usually complicated by hardening

FIG. 1–3. Construction of tissue basket.

of the paraffin if too many vials are removed from the oven at once, or by cooling of the oven itself if it is too frequently opened. The same criticism applies to the process of embedding tissues when large numbers of vials must be handled simultaneously.

The use of tissue baskets makes it possible to change the paraffin for groups of 25 tissues (or more) in a few seconds time, and the maintenance of the tissue baskets in a single paraffin bath during embedding obviates the annoyance of hardening of the paraffin.

[1]Purdue University Agricultural Experiment Station Journal Paper No. 214.

The baskets shown in Figures 1–4 were made from ordinary galvanized window screen having 16 mesh per linear inch, but can be constructed from any perforated metal. If the baskets are to be used in reagents other than paraffin, they should be of monel metal or plated with some non-corrosive metal such as chromium.

The construction is simple. A piece of screen approximately $2\frac{1}{2} \times 1\frac{1}{4}$ inches (Fig. 1) is rolled around a cylinder approximately $\frac{5}{8}$ inches in diameter. A joint is made by inserting the $\frac{1}{4}$ inch ends of the wire through the screen and folding them back (Fig. 2). This individual basket is placed on a base about $3\frac{3}{4} \times 1\frac{1}{2}$ inches (Fig. 3)

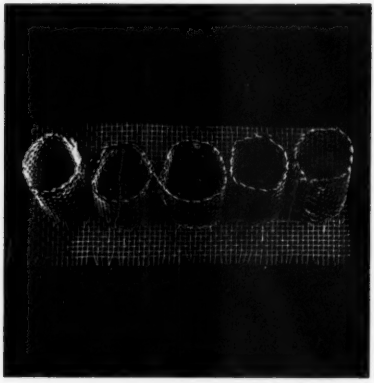

FIG. 4. Completed tissue basket.

and fastened by bending the wire ends. We have found groups of five (Fig. 4) to be most convenient for our particular purposes. Five groups of five each can be fitted into readily available enamel instrument pans $8\frac{7}{8} \times 5\frac{1}{8} \times 2$ inches in size.

When changes of paraffin are made, the baskets can be easily prevented from falling out by holding one hand over the entire group. During embedding the enamel pan can be placed on a hot plate and the paraffin can be maintained at the proper temperature. An extra pair of forceps can be placed in one of the baskets and by alternating instruments the necessity of warming forceps in a flame is often eliminated.

PREFIXING WITH PARADICHLOROBENZENE TO FACILITATE CHROMOSOME STUDY[1]

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ABSTRACT.—**A technic was developed which resulted in preparations containing many mitotic divisions with chromosomes well fixed and stained, rod-shaped, and spread throughout the cell. This technic has given good results with guayule (*Parthenium argentatum*), *Crepis*, *Allium*, *Pisum*, *Lycopersicon*, *Tradescantia*, and other plants. Material is prefixed in a saturated solution of paradichlorobenzene for 1-4 hours, fixed in 65% acetic acid (or other suitable fixative) for 12-24 hours, hydrolyzed in 10% HCl**

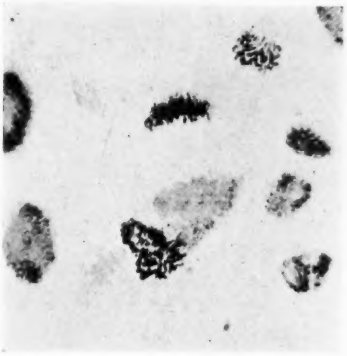

FIG. 1. Guayule chromosomes from a root tip which was not treated with paradichlorobenzene (740 ×).

for 10-30 minutes at 60° C., rinsed in water, transferred to a drop of 45% acetic acid on a slide, and smeared and stained in aceto-orcein. The preparation may be made permanent by separating slide and cover glass in 1 part glacial acetic acid to 1 part absolute alcohol, putting them in absolute alcohol, and then recombining them with a drop of euparol.

In connection with the program of the Guayule Research Project it was necessary to know something of the chromosome number and

[1]Studies leading to this paper were made while the writer was employed in the Special Guayule Research Project, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture.

chromosome morphology of guayule (*Parthenium argentatum*). The chromosomes of this plant are usually so tangled as to be difficult to study (Fig. 1). Treatment with colchicine before fixation, found advantageous in *Phlox* (Meyer, 1943), caused a clumping of the chromosomes in guayule, and so was worthless.

Simonet and Guinochet (1939) and Carey and McDonough (1943) had induced polyploidy with paradichlorobenzene. It therefore was suspected that this substance might be helpful with guayule chromosome studies.

After some trials, a technic was developed which resulted in preparations containing many divisions in which the chromosomes are well fixed and stained, rod shaped, and spread throughout the cell (Figs. 2 & 3). This technic also gives good preparations with

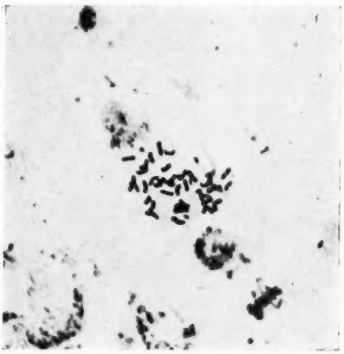

FIG. 2. Guayule chromosomes after a one hour prefixation in a saturated solution of paradichlorobenzene (740 ×).

Crepis, *Allium*, *Tradescantia*, *Pisum*, *Lycopersicon*, *Fragaria*, *Juglans*, *Taraxacum*, *Tulbaghia*, *Cooperia*, *Zephyranthes*, *Hymenocallis* and *Habranthus*.

Various active meristems have been used successfully. It is essential that the material be penetrated by the prefixative. Also the material must not have cell inclusions which will prevent the cells from being smeared or the chromosomes from being seen. The use of young ovules, anther walls, leaves, stem tips, and root tips has resulted in good mitotic preparations. Roots for smears require special cultural methods so as not to have adherent soil particles which would break cover glasses. In guayule the best results were obtained by germinating seeds on moist paper, prefixing the entire young seedlings, and using the root tips.

SCHEDULE

(1) Prefixation: The prefixative is a saturated solution prepared by mixing about 5 or 10 g. paradichlorobenzene crystals and about 500 ml. distilled water in a stoppered bottle and holding at about 60° C. overnight. It is used at room temperature.
Time: 1 to 4 hours.

(2) Fixation: Three fixatives have been used successfully with different materials —
   (a) Acetic acid, 65%.
   (b) 3 parts absolute alcohol to 1 part glacial acetic acid, used fresh.
   (c) 3 parts absolute alcohol, 1 part glacial acetic acid, 1 part chloroform.
Time: 12 to 24 hours.

FIG. 3. Chromosomes of *Crepis capillaris* after a 2 hour prefixation in a saturated solution of paradich'orobenzene (740 ×).

(3) Hydrolysis: Concentrated HCl, 10% by volume, in distilled water. Used at 60° C.
Time: 10 to 30 minutes (Stop hydrolysis by putting material through 2 or 3 changes of distilled water at room temperature.)

(4) Dissection: Transfer material to drop of 45% acetic acid on slide; find meristem; discard other tissues.

(5) Staining and smearing: Make saturated solution of aceto-orcein by bringing 45% acetic acid and synthetic orcein to a boil. Cool. Store in refrigerator. Decant or filter before using. Dilute with 45% acetic acid if lighter staining is desired. Place material in a small drop of this solution. (Slides for immediate use can be made by substituting a mixture of 8 parts aceto-orcein, 1 part Certo, 1 part Karo for the aceto-orcein. Modi-

fied from Zirkle, 1937.) Pulverize by tapping with a full spear needle. Cover with No. 1 cover glass. Hold cover glass edge with thumb and run a needle over it to smear material.

(6) Making permanent: Invert slide in petri dish containing 1 part glacial acetic acid to 1 part absolute alcohol. Slide is supported by pieces of glass so cover glass can fall to bottom of petri dish. Slide and cover glass are put in absolute alcohol, and then recombined with a drop of euparol. Care must be taken to prevent clouding. (Xylene and xylene balsam can be used after the absolute alcohol, but have given inferior results.)

Discussion

If desired, all of the operations from prefixation through hydrolysis can be carried on in one vial.

It is important that nothing happen to interfere with prefixation. The bottles, vials, corks, razor blades and forceps which are used during prefixation should be free from chemical contamination which might kill or render the material useless. In collecting material from the plant, quick cuts should be made with a sharp razor blade and the removed portions immediately submerged in the prefixative. The prefixative should approximate the plant temperature so that the material does not get a temperature shock. The material must not wilt during prefixation.

The duration of prefixation should be long enough to give many well-spread divisions with rod-shaped chromosomes, but not so long that a majority of the divisions have either very short chromosomes or have double the somatic chromosome number. The optimum duration of prefixation will differ with the type of tissue and the kind of plant and must be determined by experiment.

The fixative must penetrate and kill the material quickly, and not color the material or prevent it from smearing. It also must preserve the chromosomes so that they will stain well and have smooth and distinct boundaries and be well separated from each other. While it does not give excellent results with all materials, 65% acetic acid does give fair chromosome fixation in many plants and can be used until a better fixative is found for a particular kind of plant. Although the best slides are obtained from freshly fixed material, the material can be stored in a refrigerator in the original fixative, or can be stored at room temperature in 70% alcohol. Material which has been fixed in 65% acetic acid and is allowed to remain in it quickly loses its ability to be stained; some materials will not stain after being kept in 65% acetic acid at room temperature for a week.

Hydrolysis may be carried on in an oven regulated to 60° C. It should be carried on long enough to soften the tissues so that the cells will smear well, but not so long that the chromosomes will not stain. The optimum duration of hydrolysis will differ with different tissues and kinds of plants, and must be determined by experiment.

Small root tips may be arranged in rows and smeared without previous pulverization. The needle pressure on the cover glass should be even, and the amount needed will differ with the material. Pressure which would damage large chromosomes is usually needed to adequately spread and flatten small chromosomes.

If the cells are flattened but require deeper staining, the edges of the cover glass may be irrigated with a little stain. If this fails to get the stain to the material, the cover glass can be held down by one edge while a little stain is added and the opposite edge is carefully pried up a little. Heat is not necessary with aceto-orcein, since it rapidly destains the chromosomes and the cytoplasm is not usually stained. Iron is not used in aceto-orcein.

If a slide is to be made permanent, the cover glass edges should be irrigated with 45% acetic acid. After the precipitated stain has gone back into solution, the slide is blotted under pressure and then immediately inverted in a petri dish containing 1:1 absolute alcohol and glacial acetic acid. The slide is supported by pieces of glass under its ends so that the cover glass can fall to the bottom of the petri dish. If the cover glass has not fallen from the slide in 30 minutes, the slide should be turned right side up and the cover glass carefully pried off and placed in the petri dish, material side up.

After mounting with euparol, a small piece of blotting paper is placed on one end of the slide, along, but not over, one edge of the cover glass, to absorb excess alcohol and to hold the cover glass in place; the slide is immediately put into a warm oven. Care must be taken not to breathe on the wet slide, or it will become cloudy. After it has hardened for a few days in the oven, the excess euparol may be scraped off with a razor blade.

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A COMBINATION BLEACHING-CLEARING AGENT AND ITS USE IN THE PROCESSING OF "SPALTEHOLZ" PREPARATIONS

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In the processing of stained and cleared *in-toto* preparation of skeletal material it is often advisable to use a bleaching agent to depigment the animal before carrying out the staining process.

With small animals, such as salamanders and other small amphibians, it is almost essential that the animals be bleached because they are too small and delicate to be properly skinned. Larger forms, such as young kittens, rabbits, etc., may be processed successfully after skinning but these forms make better preparations if they are bleached to render invisible the coagulated blood and darkened areas in the skinned animal.

For a number of years various investigators have been using different percentages of commercial H_2O_2 as bleaching agents preceding the Spalteholz[1] and other methods for the preparation of stained skeletons. More delicate preparations are successfully bleached with the mixture of 3 parts of water to 1 part of H_2O_2 recommended by Reagan[2], and tougher or more heavily pigmented specimens sometimes require H_2O_2 (3% commercial) in full strength to carry out the bleaching operation. This bleaching step is carried out more rapidly in larger animals if they are first deviscerated and the eyes removed.

Following bleaching the next step, in the usual method of preparation, calls for clearing in ½%–1% KOH until the ribs are visible. Both the bleaching and the preliminary clearing steps may take several days even in small forms like *Triturus viridescens*.

A method that has been used, with excellent results, by the author consists in combining the operations of bleaching and preliminary clearing. In following this new method a 50–50 mixture of 3% H_2O_2 and 1% KOH is used and the specimen is placed in the mixture and bleached and cleared in one operation. In the preparation of more delicate specimens a 50–50 mixture of 3% H_2O_2 and ½% KOH is used. The bleaching-clearing process is carried on until the specimen is completely depigmented and the ribs, or

[1]Guyer, M. F. 1936. Animal Micrology, *4th Ed.* Univ. of Chicago Press. *See p. 117.*

[2]Reagan, F. P. 1926. Univ. of Cal. Pub. in Zool.

the bones of the legs, are visible. Sometimes it is advisable to change, or renew, the bleaching-clearing mixture every day for several days in order to obtain the desired results.

Combining these two essential steps saves days, and in some cases weeks, in the time involved in processing Spalteholz preparations. The bleaching-clearing step may be successfully used in the methods of Van Wijhe and Lundvall[3], Sabin, Cunningham, and Reagan[4], as well as those of Schultze and Mall[5], Dawson[6], Lipman[7] and others.

The bleaching-clearing step is followed by staining for bone or cartilage, destaining, clearing, and mounting.

[3]Lee, A. B. 1937. The Microtomists Vade-mecum, *10th Ed., edited by J. B. Gatenby.* Blakistons, Phila. *See p. 433.*

[4]Ibid., p. 370.

[5]Ibid., p. 434.

[6]Ibid., p. 435.

[7]Lipman, H. J. 1935. Staining the skeleton of cleared embryos with alizarin red S. Stain Techn., **10**, 61.

THE USE OF CERTAIN PHTHALATE COMPOUNDS IN EMBRYOLOGY

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Certain phthallic acid derivatives have proven advantageous in the preparation of embryos for study and observation. The information is therefore described.

A. *Dimethoxy ethyl phthalate*[1] $C_6H_4(COOC_2H_4OCH_3)_2$

This solution has been used mainly to clear dog-fish, chick and mammalian embryos for class study. It has a refractive index of 1.500 at 25° C., a molecular weight of 282, and a solubility in water of 0.838. It will displace 85% or 95% alcohol in tissues. Tissue may be left in it indefinitely. Consequently, as an intermediate step between alcohol and benzene, chloroform or xylene in ordinary paraffin technic, it is useful. It is a suitable medium for the whole preparation study of various vertebrate embryos, and is particularly serviceable in the study of stages of early chick development.

For chick embryos it has been used as follows:

1. To displace 95% alcohol or dimethoxytetraethyleneglycol. In doing so a thin layer of either of these solutions is placed upon the dimethoxy ethyl phthalate and the embryo allowed to sink into the phthalate solution. The embryo may then be removed by means of a spatula to a new phthalate solution in a small stender dish and studied by the student as a whole mount preparation. Its refractive index of 1.500 permits of considerable clarity, and the student is able to move the embryo at will, thus observing different aspects in study. As is true in ordinary whole mount preparations, the embryos should not be too heavily stained. Lightly stained embryos prove best.

2. If desired, after the student has studied such a preparation, the embryo may be placed in equal parts of benzene and dimethoxy ethyl phthalate, then into pure benzene, two changes, and thence into paraffin and sectioned. Or the embryo may be gradually transferred into xylene and a permanent whole mount may be made.

3. Embryos (dog-fish, frog, chick, etc.) may be left in dimethoxy ethyl phthalate indefinitely, stained or unstained, and used as above when convenient.

[1]Courtesy of Ohio Apex, Inc., Nitro, W. Va.

B. *Dimethyl phthalate*[2] $C_6H_4(COOCH_3)_2$

This solution has a molecular weight of 194, a refractive index of 1.513 at 25° C., and a solubility in water of 0.45. It may be used similarly to dimethoxy ethyl phthalate. Embryos appear to soften slightly in this fluid, and when used for study of whole embryos, the student should take care in turning the embryo over in the solution.

C. *Dibutyl phthalate*[2] $C_6H_4(COOC_4H_9)_2$

This solution has a molecular weight of 278 and a refractive index of 1.490 at 25° C., and a water solubility of 0.03. It may be used similarly to the two phthalate solutions previously mentioned. Small dog-fish embryos, unstained or lightly stained, show to great advantage in this solution. The author has kept embryos for class study in this solution for various periods up to five years.

D. *Diethyl phthalate* $C_6H_4(COOC_2H_5)_2$

This solution has a molecular weight of 222, refractive index of 1.499 at 25° C., and a solubility in water of 0.09. Embryos appear much the same in it as in dibutyl phthalate.

[2]Courtesy of I. E. du Pont de Nemours & Co., Phila., Pa.

THE USE OF DIMETHOXYTETRAETHYLENEGLYCOL AND TRIETHYL PHOSPHATE IN HISTOLOGICAL TECHNIC

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For a considerable period of time the author has used these two compounds in histological technic. As they have proved valuable, the information is described.

I. *Dimethoxytetraethyleneglycol*[1] (Dimethoxytetraglycol) (Tetraethylene glycol dimethyl ether) $O(CH_2CH_2OCH_2CH_2OCH_3)_2$

This is a stable liquid with a flash point of 285° F. and a molecular weight of 222. It has five ether groups in its molecule; as a result it possesses excellent solvent power for many substances. Its volatility is low, with a boiling point of 275.8° C. Despite its high molecular weight it is soluble in water to infinity. It has a slight "butyl alcohol" odor.

A. In histological technic it has been used mainly to displace water or alcohol in tissues in any strength. Water may be removed after fixation by placing tissue directly into this fluid. The writer has used it also to displace such fixatives as Bouin's and its various modifications, and Fleming's fluid. However, it appears preferable to first wash in water. Generally, it has been used as follows:

1. Fixation of tissue.
2. Wash in water for 20 minutes.
3. Place tissue in dimethoxytetraethyleneglycol for several hours or overnight in glass, metal, or cork stoppered container. The writer has made a practice of the latter procedure, using two changes of dimethoxytetraethyleneglycol.
4. Place in equal parts of dimethoxytetraethyleneglycol and thiophene-free benzene or in chloroform for 15–20 minutes. (Toluene or xylene also may be used if preferred.)
5. Full strength benzene or chloroform for 15 minutes.
6. Full strength benzene or chloroform for 15 minutes.
7. Paraffin, two changes; embed. Tissue may be placed from it directly into paraffin, but the intermediate step of benzene or chloroform appears preferable.

The advantages of this fluid are as follows: (1) Tissues may be left in it for several days to several weeks without injury. (2) Possibly due to the fact that its molecules are large, it displaces water

[1]Courtesy of Carbide-Carbon Chemicals, New York, N. Y.

in tissue gradually; hence distortion or shrinkage is slight, and a tedious displacement series is not necessary. (3) It is stable and possesses a low volatility.

B. For chick embryos it may be employed as follows:
   1. After fixation and washing, stain the embryo *in toto* in alum cochineal, borax carmine, etc.
   2. Destain with HCl-water until proper staining is attained.
   3. Neutralize excess HCl with water in which $NaHCO_3$ has been dissolved.
   4. Place in dimethoxytetraethyleneglycol and proceed as above for sectioning. If it is desired to mount the preparation without sectioning, it should be placed in dimethoxytetraethyleneglycol. Then proceed as follows:
      (1) Place in equal parts of dimethoxytetraethyleneglycol and xylene for 20 minutes.
      (2) One part dimethoxytetraethyleneglycol and three parts xylene for 15 minutes.
      (3) Pure xylene.
      (4) Mount in the usual way.

C. For slide preparations stained with hematoxylin or other nuclear stains proceed as follows:
   1. After staining and washing use two changes of dimethoxytetraethyleneglycol.
   2. Xylene, two changes.
   3. Mount.

II. *Triethyl phosphate*[2] $(C_2H_5O)_3PO_4$

Triethyl phosphate has a molecular weight of 182. It has a boiling range of 210–220° C., a refractive index of 1.4055 at 20° C. and is soluble in water to infinity. It has a slight, pleasant odor. This solution may be used in ordinary paraffin technic, as it displaces water in tissues readily with slight, if any, shrinkage or distortion. Tissue may be placed directly into it from water. It is soluble in the alcohols, benzene, ether, chloroform and xylene. It is excellent for smear preparations. After fixation and staining, the smear can be transferred to equal parts of water and triethyl phosphate, to pure triethyl phosphate and thence to xylene and mounted. It has proved to be an excellent solution following the Feulgen reaction, as it does not extract the stain. Fast green or light green may be dissolved in it for counterstaining purposes. Small objects such as hydra are readily transferred through it to xylene; the author has mounted green hydra permanently with slight loss of the chlorophyll coloring.

[2]Courtesy of Eastman Kodak Co., Rochester, N. Y.

THE VENETIAN TURPENTINE MOUNTING MEDIUM

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Abstract.—**As a mounting medium to follow aceto-carmine the following modification of Zirkle's is suggested: Venetian turpentine, 25 ml.; phenol, 50 ml.; propionic acid, 35 ml.; acetic acid, 10 ml.; water, 20 ml. The technic can be employed with either root-tip or pollen-mother smears, and has been used with quite a variety of plants. It is especially valuable where it is desired to make temporary mounts permanent. The method is simple, and with reasonable care no displacement of marked cells occurs.**

The desirability of finding a mounting medium which can be used with aceto-carmine without the necessity of going through the numerous steps of the McClintock method has been recognized by numerous workers. In particular, Zirkle (1937, 1940) has made exhaustive studies on a number of fluids which can be combined with aceto-carmine to give a series of media which are in effect combined fixing-staining-mounting media, making it possible to go from fresh material to permanent mounts in a single step thus saving both time and material. Three of these methods, namely the Certo-Karo, the Canada-balsam-oleic-acid and the Venetian Turpentine technics were communicated to the writer by Dr. B. R. Nebel prior to Dr. Zirkle's publication and were tried out on *Tradescantia* microspores. Of these three methods the Venetian Turpentine proved to be by far the best and has been used extensively on various materials since 1939 by the writer and his associates. Combination of the stain with the other ingredients has been found to be unsatisfactory in most cases owing to the difficulty of obtaining sufficiently intense staining. The technic as finally established consists therefore of three main steps: (1) fixation in acetic-alcohol plus masceration in HCl or 45% acetic acid if necessary; (2) staining with strong aceto-carmine and (3) replacement of the stain with the Venetian Turpentine mixture.

Following is a detailed schedule for pollen mother cells which differs only slightly from that originally suggested to the writer by Dr. Nebel.

1. Squeeze the cells from fresh anthers onto a slide covered with a thin film of the mounting medium.
2. Cover the material with a drop or two of 3 parts absolute alcohol to 1 part glacial acetic acid for 1 to 2 minutes.

3. Decant the fixative and cover with as small an amount of stain as possible. Heat gently and allow to stain for 3 to 5 minutes.
4. Remove stain by flooding gently with 45% acetic acid.
5. Replace the acetic acid with the following mounting medium:—

	Zirkle	*Wilson*
Venice Turpentine	20 ml.	25 ml.
Phenol	50–60 ml.	50 ml.
Propionic acid	40 ml.	35 ml.
Acetic acid	15 ml.	10 ml.
Water	25 ml.	20 ml.

6. Cover, heat to near boiling and press.

The only step in which any difficulty is likely to be encountered is in the replacement of the acetic acid (or carmine) with the mounting fluid. At the line of contact an emulsion is formed which is very persistent. This trouble can be largely avoided by placing a drop of the mounting fluid alongside the acetic acid rather than on top. The result of this is that the acid is pushed aside by the mounting medium. If an emulsion is formed, reapply the mounting fluid until it is clear.

A similar method for use with root-tips has been devised. This as used by Wilson and Boothroyd (1941, 1944) is as follows:—

1. Fix in 3:1 at 60°C. for ½ to 1 hour.
2. Mascerate in 45% acetic acid at 60°C. for ½ hour.
3. Squash root-tips in a drop of aceto-carmine on a slide covered with a thin film of the mounting medium and heat gently.
4. Replace stain with mounting fluid as described above.
5. Cover, heat and press.

Slides made by this method are at first semi-permanent and must be watched for several weeks to prevent drying out. Ultimately, however, they will harden and become as firm as balsam-mounted slides. The writer's version of the formula has a slight advantage over Dr. Zirkle's formula in that drying out occurs less often and the hardening process takes less time. Otherwise, there does not seem to be much difference between them.

The slides made in 1939–40 of *Tradescantia reflexa* microspores were re-examined in detail in 1942 and found to be perfectly preserved with no fading or cloudiness. With some materials, especially meiotic divisions of *Tradescantia* and to a lesser extent with *Trillium* root tips, this medium performs the useful function of removing most of the stain from the cytoplasm without apparently affecting that of the chromosomes, thus greatly improving the differentiation.

This technic has been used with considerable success on a variety of materials, especially the following: meiotic divisions of *Tradescantia reflexa;* root tips and meiotic divisions of several species of *Trillium;* root tips of rye; pollen mother cells of wheat and salivary glands of *Drosophila.* In the case of *Trillium* root tips and *Tradescantia* microspores slides have remained in perfect condition for three and four years.

Some experiments were conducted on *Allium* root tips to test the efficacy of this medium after Feulgen treatment. The slides were only retained for two or three months, but during that time they showed no tendency to fade or otherwise deteriorate.

This medium has been found to be especially valuable where it is desired to make temporary mounts permanent. The technic is exceedingly simple and with reasonable care no displacement of marked cells occurs. The coverslip is forced up gently at one corner with a thin razor blade and a drop of the mounting fluid allowed to run underneath. If left for a few minutes with the razor blade still inserted, the fluid will push out the aceto-carmine or 45% acetic acid and completely replace it. When replacement is complete, the razor blade is removed carefully and the mount allowed to harden. Excess fluid may be cleaned off with xylene. If the razor blade is not removed until complete replacement, no emulsification will occur. The process may be hastened by pressing gently just behind the line of junction between carmine or acid and the mounting fluid.

This technic is currently being employed with both root tip and pollen mother smears of *Musa.* Although there appears to be some tendency to fade in the Tropics, most slides have remained serviceable for upwards of four months.

The optical qualities of the Venetian Turpentine method are not as good as the best balsam but by and large are satisfactory for general work and where the film is very thin, as in most pollen mother cell smears, quite satisfactory for detailed studies of chromosome structure.

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NOTES ON TECHNIC

A Device to Aid Paraffin Embedding[1]

Numerous types of containers have been described for forming paraffin blocks during embedding. Each has its adherents and each its disadvantages. The necessity for rapid cooling of the paraffin to prevent crystallization is well recognized and this is usually accomplished by placing the container in cold water. Most containers have the common disadvantage that they must be maintained above water until a film of paraffin is formed on the surface. Carefully constructed paper boats will often float without capsizing while the paraffin cools but most technicians usually hold the boat until it is safe from water damage. This interval of waiting can be elimin-

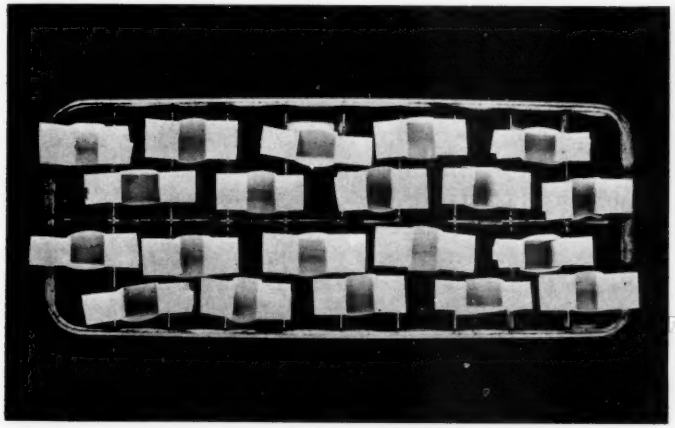

Fig. 1. The use of an ice cube tray for cooling paraffin blocks.

ated by the use of an ordinary electric refrigerator ice cube tray. The flaps of the boats can be rested on the edges of the rack in the tray (Fig. 1) as soon as the tissue has been oriented in the paraffin. The boat sinks in the water nearly to the top and cooling occurs rapidly and without danger of water penetration. A device of this type doubles the speed of embedding and makes it an efficient "one man" operation. We have found that an initial water temperature of 10° C. is satisfactory for rapid cooling of the paraffin with minimum shrinkage and an absence of crystallization.—Frederick N. Andrews, *Department of Animal Husbandry, Purdue University, Lafayette, Indiana.*

[1]Purdue University Agricultural Experiment Station, Journal Paper No. 215.

Method for Affixing Celloidin Sections

Because celloidin absorbs some stains, as methyl green, the celloidin must first be removed from the tissue before such stains can be satisfactorily employed. This is done by first affixing the section to a slide and then dissolving the embedding medium. The usual technic for this involves the use of egg albumin affixative, the dissolving away of the celloidin in clove oil, and then washing in absolute alcohol. This is a slow and relatively costly process and seldom works on sections that contain air bubbles or are in any way not intact.

An alternate method which may at times prove useful is as follows: To a clean slide a drop of Haupt's affixative[1] is added and quickly rubbed out. The section to be mounted is removed from 70% alcohol on a cigarette paper and is then transferred to a slide where a folded filter paper is used to blot and firmly press it. The cigarette paper is then carefully rolled off the section which is left adhering to the slide. All this should be done rapidly, and the section should not be actually dried. The slide is then placed immediately in a Coplin jar containing 50-50 ether-alcohol and allowed to remain until the celloidin is dissolved. This usually takes from ten to twenty minutes. The procedure appeared to have no untoward effect upon tissues which had been fixed in Zenker-Formol. As soon as the celloidin has dissolved, the slide is removed to another Coplin jar containing 70% alcohol. Whatever stain procedure is chosen can now be employed. Care must be taken in transferring the slides; however, most will stand a considerable amount of manipulation.

This method is not perfect because an occasional section may come off. But because of its speed and ease the method is more satisfactory than the standard procedure.—Louis W. Lewis, *442 Woodbury Rd., Glendale, Cal.*

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[1]Haupt's affixative:

	grams
Knox gelatin	7.0
Distilled water	42.0
Soak for two hours	
Glycerin	50.0
Phenol crystals	0.5

Heat ten to fifteen minutes in water bath.

Filter while hot through glass wool. Store by sealing in 5 or 10 ml. test tubes.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

DUBOS, RENÉ J. **The Bacterial Cell.** 5½ × 8½ in. 460 pp. Harvard Univ. Press, Cambridge, Mass. 1945.

This book is unlike any other that has yet made its appearance. The author's attention is not confined wholly to bacterial cytology as the title, as given above, would suggest. He also discusses various phases of bacteriology in relation to the cell; this idea is brought out by the subtitle "in its relation to problems of virulence, immunity, and chemotherapy." The book is based partly on the author's own work and partly on a review of the literature. Possibly some of the results in the literature are accepted somewhat too uncritically; the reviewer has noticed, for instance, several places where work of his own or of an associate has been misinterpreted, apparently due to hasty reading of the original. In spite of such slips the book is thought-provoking, and calls for serious reading.

The author devotes a 35–40 page chapter to each of the following subjects: cytology of bacteria; physicochemical and staining properties of bacteria; analysis of cellular structure by biochemical and biological methods. A slightly longer chapter takes up the variability of bacteria. The rest of the main part of the book (three chapters) is given up to the subject matter particularly specified in the subtitle: virulence, immunity and chemotherapy. The whole book is well illustrated with tables, photomicrographs and other illustrations, some original and some borrowed from other authors. There is a bibliography of some 1100 references.

At the back of the book is an "addendum" by C. F. Robinow on "Nuclear Apparatus and Cell Structure of Rod-shaped Bacteria," an original contribution of about 25 pages in length. This paper, which includes numerous photomicrographs, presents strong indication of true nuclear division by certain bacteria. This contribution, however, is only incidentally mentioned by Dr. Dubos in the main part of the book.—*H. J. Conn.*

MACKIE, T. J. and McCARTNEY, J. E. **Handbook of Practical Bacteriology.** *7th Ed.* 5 × 7½ in. 720 pp. Williams and Wilkins Co., Baltimore, Md. 1945. $5.00.

This is a well known bacteriological text by two British authors, probably much more familiar to bacteriologists on the other side of the Atlantic than in America. It is outside the field of books generally reviewed in this journal, but deserves mention here, nevertheless, because of a chapter of some forty-odd pages on Staining Methods. Methods are in general adequately presented, but in many cases represent procedures less familiar to Americans than those standard in this country; as a matter of fact they are all the more interesting and valuable for that very reason. Significantly one or two recent American modifications of staining methods that have become well established in this country (e.g. the Kopeloff-Beerman modification of the Gram stain and the Laybourne modification of the Albert diphtheria stain) are given by these British authors.—*H. J. Conn.*

DYES AND THEIR BIOLOGICAL USES

ALBERT, ADRIEN, and MAGRATH, DAVID. **Improved syntheses of aminoacridines. Part III. 1:9-Dimethylproflavine.** *J. Soc. Chem. Ind.*, **64**, 30. 1945.

A method of preparation and purification of 1:9-dimethylproflavine (2:8-diamino-1:9-dimethylacridine; suproflavine) which provides larger quantities of material for clinical trials is described. In laboratory tests now in preparation this substance has been found to exceed considerably, in activity evinced against *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Clostridium welchii*, the aminoacridines hitherto available.—*A. P. Bradshaw.*

BAMBAS, L. L. **Some chemotherapeutically active sulfones. I.** *J. Amer. Chem. Soc.*, **67**, 668. 1945.

A series of 4-aminophenylaminoheterocycle sulfones, analogs of 4, 4′-diaminodiphenyl sulfone, has been made with the object of obtaining compounds having high antistreptococcal and antipneumococcal activity and low toxicity. Only those compounds which had at least one benzene nucleus with nitrogen in the para position to the sulfur were effective. Compounds in which the pyridyl, thiadiazolyl and quinolyl rings were substituted for one of the phenyl rings retained high antistreptococcal activity but not as great as 4,4′-diaminodiphenyl sulfone. All the compounds were less toxic than 4, 4′-diaminodiphenyl sulfone. (Toxicity was tested by oral feeding of white mice. β hemolytic streptococcic infections and Type I pneumococcic infections were used. Tuberculo-therapeutic activity of these compounds will be reported by W. H. Feldman and H. C. Hinshaw, Mayo Foundation and Mayo Clinic.)—*A. P. Bradshaw.*

BAMBAS, L. L. **Some chemotherapeutically active sulfones. II. 4-Aminophenyl-2′-aminothiazolyl-5′ sulfone and analogs.** *J. Amer. Chem. Soc.*, **67**, 671. 1945.

The chemotherapeutic activity shown by a series of 4-aminophenylamino heterocycle sulfones led to further investigation of other members of the series. 4-Aminophenyl-2′-aminothiazolyl-5′ sulfone ("Promizole") has been prepared and was shown by Feldman, Hinshaw and Mann to have tuberculotherapeutic activity for a human strain of the tubercle bacillus in the guinea pig and to have a low degree of human toxicity. This compound and several others of the series are shown to have antistreptococcal activity.—*A. P. Bradshaw.*

CHU, EDITH JU-HWA. **Syntheses of compounds related to vitamin K. III. 4-(3′-Methyl-4′-hydroxynaphthylazo)-benzenesulfonamide and related compounds.** *J. Amer. Chem. Soc.*, **67**, 811. 1945.

Synthesis of 4-(3′-methyl-4′-aminonaphthylazo)-benzenesulfonamide, 4-(3′-methyl-4′-hydroxynaphthylazo)-benzenesulfonamide and their analogs derived from albucid, sulfapyridine, sulfathiazole, sulfadiazine and sulfaguanidine is reported and the preparation of corresponding acetyl derivatives. Antihemorrhagic activities and antibacterial effects of these compounds are being studied and will be reported elsewhere. A preliminary test shows that 4-(3′-methyl-4′-hydroxynaphthylazo)-benzenesulfonamide possesses an antihemorrhagic activity almost equal to that of 2-methyl-1,4-naphthoquinone.—*A. P. Bradshaw.*

KLOTZ, IRVING M., and GUTMANN, HELMUT R. **The mode of action of sulfonamides. Dissociation constants of the enzyme-drug complex.** *J. Amer. Chem. Soc.*, **67**, 558. 1945.

The rates of growth of *Escherichia coli* have been followed in the presence of sulfanilamide, sulfathiazole, sulfapyridine and N′-benzylosulfanilamide. An analysis of the growth curves indicates that competitive inhibition is obtained in each case. Dissociation constants for the enzyme-drug complexes have been calculated and their relation to bacteriostatic activity discussed.—*Author's Summary.*

LILLIE, R. D. **A Giemsa stain of quite constant composition and performance, made in the laboratory from eosin and methylene blue.** *Publ. Health Repts.* **58**, 449–52. 1943.

The variability among Giemsa stains is due to lack of uniformity in the azure components. Azures of more uniform composition than those procurable commercially were made by the oxidation of methylene blue with specified amounts of $K_2Cr_2O_7$ in acid solution with boiling, followed by immediate cooling and neutralization with precalculated amounts of $NaHCO_3$. These were promptly precipitated with eosin, filtered and dried. Three eosinates are thus prepared, and these are ground separately and mixed in a definite proportion for the final stain.—*Lois Almon.* (Courtesy *Biological Abstracts*).

STROCKTON, J. RICHARD, and LOCHTE, H. L. **Some naphthenyl sulfanilamides.** *J. Amer. Chem. Soc.*, **67**, 160. 1945.

The author reports preparation of acylsulfanilamides from cyclopentanecarboxylic, campholic and 2,2,6-trimethylcyclohexanecarboxylic acids and some of their properties are described. Some of these compounds were moderately or slightly effective in streptococcic infections in mice but were ineffective in pneumococcic or meningococcic infections. Low toxicity was exhibited.—*H. J. Conn.*

ANIMAL MICROTECHNIC

MARGOLIS, GEORGE. **The diagnosis of granuloma venereum from frozen sections stained with polychrome methylene blue.** *Amer. J. Path.*, **21**, 543–7. 1945.

Tissue is obtained from lesions clinically resembling granuloma venereum and fixed in formalin. Frozen sections, 20 to 25 μ in thickness, are then cut and floated on, or dipped into, Terry's neutralized polychrome methylene blue stain for a few seconds. Following staining, the sections are washed and mounted on a slide in water. Tissues stained by this method cannot be satisfactorily preserved but they may be kept long enough to allow careful study. The author believes that the details in the morphology of the Donovan bodies, especially the capsular zone, are demonstrated better with this technic than by any other section method. The author lists in detail the steps in the preparation of Terry's neutralized polychrome methylene blue.—*J. O. Foley.*

PLANT MICROTECHNIC

LUTMAN, B. F. **Actinomycetes in various parts of the potato and other plants.** *Vt. Agr. Expr. Station, Bull.* 522, 1–72. 1945.

The writer gives some attention to staining methods in trying to establish his thesis that *Actinomyces* filaments occur throughout the tissue of normal potatoes and fleshy roots of other plants, but have ordinarily been interpreted as middle lamellae of cell walls. The greater number of his staining procedures are taken from the work of other writers, but he gives particular attention to his own modification (Hutchins and Lutman, STAIN TECHN., **16**, 63–6, 1941) of the Gram stain. Of special interest is his observation that he can thus employ crystal violet as a microchemical indicator of reaction, due to the fact that it has two ranges of color change: from yellow to blue-violet at about pH 2; and from blue-violet to violet between pH 5 and pH 6. By this means he shows that the filaments under consideration have a pH of about 5 (which is known to be about that of fungus hyphae), while parenchyma tissue is regarded as being around pH 6.0 to 6.2.—*H. J. Conn.*

MICROÖRGANISMS

GAGE, S. H. **Zymogen granules of the pancreas in vertebrates.** *Trans. Amer. Micr. Soc.*, **64**, 151–81. 1945.

This paper emphasizes the necessity for elimination of alcohol from fixatives for pancreas when zymogen granules are to be studied. The granules can be isolated by teasing fresh tissue in a dissociating liquid consisting of 1 liter of normal salt solution plus 2 ml. strong formalin, or by crushing with a scalpel tissues placed in 20% HNO_3 for 2 to 3 days at room temperature and stored in alum water after they have been rinsed in water. The isolated granules on the slide are placed in glycerol to which 0.5% acid fuchsin has been added. The granules appear to best advantage in dark field.— *Virgene Kavanagh.*

LEMPERT, HYME. **Fluorescence microscopy in the detection of tubercle bacilli.** *Lancet* **247**, 818–22. 1944.

Fluorescence microscopy, the apparatus required, and the application of the method to the detection of tubercle bacilli, are described. Smears of sputum or centrifuged urine deposits are made on 3 × 1 slides and fixed with heat. The slides are stained with auramine-phenol (100 ml. 3% phenol warmed to 40°C and 0.3 g. auramine O added gradually with vigorous shaking, filtered, keeps at least 3 weeks) for 8–10 min. After washing with tap water, acid-alcohol de-

colorizer (0.5 ml. conc. HCl, 0.5 g. NaCl, 75 ml. methanol, 25 ml. H_2O) is applied in 2 lots of 2 min. each, followed by washing in tap water. A 0.1% solution of $KMnO_4$ is applied for 20 sec. and the slide washed with tap water, blotted dry and allowed to air dry. The slides can then be irradiated with the ultra-violet light and the fluorescence observed. The tubercle bacilli may be spotted in this way with a ⅔-in. or ¼-in. objective with an 8 × eyepiece, using the 1/6 objective for confirmation. The method is more rapid and gives more positives than the Ziehl-Neelson method.—*H. Leverne Williams.* (Courtesy *Biological Abstracts*).

SYVERTON, JEROME T. and THOMAS, LEWIS. **A method for staining Rickettsia orientalis in yolk sac and other smear preparations.** *Proc. Soc. Exp. Biol. & Med.*, **59**, 87–9. 1945.

This organism is more difficult to stain than other Rickettsiae. Yolk-sac preparations, preferably washed in 0.85% NaCl, are thoroughly dried on slides. Slides are immersed in Carnoy's fixative, 3 parts chloroform, 6 parts absolute ethyl alcohol, and 1 part glacial acetic acid, for 15–30 min. After drying in air, they are stained in a Giemsa stain of 39 ml. distilled water, 4 drops $NaHCO_3$, and 1 ml. concentrated Grübler Giemsa stain. They are removed, dried, and examined.—*M. S. Marshall.*

HISTOCHEMISTRY

GOMORI, GEORGE. **The microtechnical demonstration of sites of lipase activity.** *Proc. Soc. Exp. Biol. & Med.*, **58**, 362–4. 1945.

The recommended procedure is as follows: Fix thin slices in ice cold absolute acetone 12–24 hr.; dehydrate at room temperature in two more changes, 6–12 hr. each. Embed in paraffin through 2 changes of benzene, 30 min. each, under 60° C. Cut at around 5μ, pass through xylene and alcohol, dip in 0.5% collodion in alcohol-ether, and pass through another alcohol. Incubate 6–12 hr. at 37° C. in the following solution: 5 ml. of 2% Tween 40 or 60 (Atlas Powder Co., Wilmington, Del.), 20 ml. *M*/10 maleate pH about 7.4, 20 ml. 30% glycerol, 5 ml. 2% $CaCl_2$. The solution may be slightly hazy; sites of lipase activity become opaque due to precipitation of Ca palmitate or stearate. Rinse in distilled water, transfer to 2% Pb $(NO_3)_2$ to make lead instead of calcium salts, rinse again in many changes, and transfer to yellow ammonium sulfide, 10 drops to a Coplin jar of water. Slides are finally washed in tap water, counterstained with hematoxylin, dehydrated, and mounted. Ligroine or dichloroethylene is used for clearing, clarite or a similar resin for mounting, to avoid fading due to xylene.—*M. S Marshall.*

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